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Relationship between microbial community, operational factors and ammonia inhibition resilience in anaerobic digesters at low and moderate ammonia background concentrations

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HIGHLIGHTS

- Ammonia inhibition resilience was studied for 13 inocula from distinct digesters
- Digesters substrate and temperature influenced microbial community composition
- Ammonia inhibition KI_{50} varied moderately among the inocula 32-175 $mgNH_3\text{-}N \cdot L^{-1}$
- No microbial or operational factors correlated with ammonia inhibition resilience
- Methanogenic activity was significantly correlated with archaeal relative abundance

ABSTRACT

The relationship between anaerobic digestion operational conditions and (i) microbial community, (ii) acetoclastic methanogenic activity and (iii) free ammonia (NH_3) inhibition resilience was investigated. Thirteen inocula were obtained from full and pilot scale digesters

fed with different substrates, digester configurations, operating temperatures and NH_3 concentrations (0.1 – 241 $mgN \cdot L^{-1}$). Substrate type and temperature were the primary factors influencing microbial community composition. Methanogenic activity ranged from 0.04 to 0.14 $gCOD\text{-}CH_4 \cdot g^{-1}VS \cdot day^{-1}$, and was significantly correlated with archaeal relative abundance and archaeal community PC2. The variability of NH_3 resilience among inocula was moderate, with inhibition threshold values (KI_{50}) ranging between 32 and 175 $mgNH_3\text{-}N \cdot L^{-1}$. No microbial or operational factors correlated with NH_3 resilience. However, the slopes of inhibition threshold curves were influenced by some environmental factors, namely substrate type, digester temperature and NH_3 concentration. Overall, these results indicate that low and moderate background NH_3 concentrations is not a key determinant of microbial community nor NH_3 resilience.

KEYWORDS

Anaerobic digestion; inhibition; toxicity; nitrogen; microbial community; acetoclastic methanogenesis

INTRODUCTION

Anaerobic digestion (AD) is a widely applied technology to treat industrial and domestic waste and wastewater while recovering energy in the form of biogas [1, 2]. However, the performance and stability of AD processes is sensitive to multiple environmental factors including pH, temperature, the presence of macro and micronutrients and inhibitors [3, 4].

Ammonical nitrogen is a product of the degradation of protein, amino acids, urea and nucleic acids during AD. Total ammonical nitrogen (TAN) is a key nutrient for bacterial growth, with

concentrations around 200 mgN·L^{-1} reported as sufficient to prevent nitrogen deficit, although the need for TAN may vary depending on substrate composition and loading rate [5-7]. TAN is also one of the most common inhibitors leading to poor performance in industrial, municipal and especially agri-industrial anaerobic digesters [8-10]. TAN concentration in the liquid phase consists of free ammonia nitrogen (NH_3) and ammonium ion (NH_4^+), with the relative proportions of NH_3 and NH_4^+ depending mainly on pH and temperature [11, 12]. NH_3 is a stronger inhibitor than NH_4^+, however high NH_4^+ concentrations can also contribute to inhibition [8, 13]. Reported concentrations of NH_3 resulting in inhibition and the extent of inhibition by NH_3 vary greatly, and this variability has been linked to several factors including digester substrate type, digester configuration, experimental techniques, microbial community and microbial acclimation [4, 8]. The mechanisms proposed for NH_3 inhibition of methanogens are (i) limiting expression of key enzymes in methanogenic metabolism [14] and (ii) changing intracellular pH when diffused into a cell, leading to proton imbalance and/or potassium deficiency [15].

Acetoclastic methanogens, particularly *Methanosaeta*, are often considered to be most sensitive to inhibition; possibly due to the higher surface-to-volume ratio which facilitates NH_3 diffusion [16-18]. For this reason, resilience of a microbial community to NH_3 inhibition has commonly targeted the specific methanogenic activity (SMA) across a range of inhibitor concentrations [19-21]. Inhibition response can be characterised by the profile of decreasing activity with increasing inhibitor concentration. A key inhibition indicator is the KI_{50} inhibition coefficient, that is, the concentration at which the measured activity is half the maximum measured uptake rate [11, 20]. Different levels of inhibition (KI_{50}) and inhibition profiles (e.g. threshold, progressive) have been observed for NH_3 inhibition on methanogenesis [18, 22, 23]. This variability in observed inhibition behaviour is attributed to the complexity of the anaerobic

digestion process, where multiple environmental (e.g. digester type, substrate, pH and temperature) and microbial factors (e.g. microbial profile and microbial activity) could influence inhibition resilience. There is a need to understand the relationship and contribution of environmental factors and microbial factors to NH_3 resilience, to inform future designs and operation of anaerobic digesters for improved inhibition resilience.

There is evidence in the literature that microbial acclimation leads to enhanced NH_3 resilience [8, 24-26]. Acclimation of microbial communities to high NH_3 has been primarily attributed to a change in methanogenic pathways (from acetate cleavage to syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis). This could be due to individual organisms shifting pathways [14] or alternatively could be due to the transition to more resistant microorganisms with different metabolic capability [26, 27].

NH_3 inhibition have been intensively studied in lab-scale; however, larger scale anaerobic digesters provide effective acclimation environments with varying environmental factors, including TAN and NH_3 concentrations [7, 18, 28]. The objective of this study is to examine the relationships between digesters process conditions and (i) the microbial community composition, (ii) the acetoclastic methanogenic activity of the microbial community, and (iii) NH_3 inhibition resilience. The results discussed here contribute towards the understanding of the key mechanisms behind NH_3 resilience.

MATERIALS AND METHODS

Inocula origin

13 different anaerobic inocula were tested in this study; 10 inocula were collected from full-scale anaerobic treatment plants and 3 from pilot-scale reactors (>100 L). Except the leachbed

reactors, which was operated in batch mode [29], the rest of the digesters have been operating under similar conditions for multiple hydraulic retention times. As listed in Table 1, the inocula comprised samples from anaerobic digesters fed with a variety of substrate types, operating with different digester configurations and operating temperatures. 5 L of each inoculum was collected from each site in sealed plastic drums and transported to the testing laboratory within 1-3 hours, with the exception of Slurry_1 and Slurry_3 that were instead cooled for transport (using ice bricks) and received within 36 hours after collection.

Chemical analytical methods

To measure the characteristics of the inocula as close to the host environment as possible, chemical analyses were done immediately on arrival at the laboratory. pH and TAN values may have slightly drifted during transportation. Inocula transportation may also have affected other parameters such as microbial community and the specific methanogenic activity, but such effects were minimised as much as possible by expediting the transport of samples from site to the laboratory.

Analyses of total fraction were performed directly on the raw samples, and included total chemical oxygen demand (tCOD), total solids (TS), volatile solids (VS) and pH. For analyses of the soluble fraction, sub-samples were centrifuged at 2,500 × g for 5 minutes and the supernatant filtered at 0.45 μm (PES Millipore®) and included soluble chemical oxygen demand (sCOD), TAN and phosphate. TS and VS were measured according to Standard Method 2540G [30]. tCOD and sCOD were measured using a Merck COD Spectroquant® test kit and a Move 100 colorimeter (Merck, Germany). pH was measured with a TPS WP-80D multi-parameter meter equipped with a TPS-121210 micro pH sensor. TAN and phosphate were determined with a Lachat Quik-Chem 8500 flow injection analyser following the

manufacturer's protocol. NH_3 concentration was calculated by means of equation 1. The inhibition testing TAN concentration includes the TAN contributions of the added chemical (ammonium chloride) and the inoculum background, while the pH used in Equation 1 is that measured just before the headspace of each inhibition test bottle was flushed with N_2 (see Section 2.4).

$$NH_3 = \frac{K_a \cdot TAN}{K_a + 10^{-pH}} \quad \text{(Eq. 1)}$$

The acid-base equilibrium constant for ammonia (K_a) was corrected to the assay temperature (T, in Kelvin degrees) using the Van't Hoff equation (Equation 2).

$$K_{a\,(T)} = K_{a\,(298.15)} \cdot e^{\left(\frac{51965}{8.314} \cdot \left(\frac{1}{298.15} - \frac{1}{T}\right)\right)} \quad \text{(Eq. 2)}$$

Biogas composition (CH_4, CO_2, H_2, and N_2) was determined using a Shimadzu GC-2014 equipped with a HAYESEP Q 80/100 packed column, a thermal conductivity detector (100 °C) and Argon as carrier gas (28 mL·min^{-1}).

Microbial analysis

Sub-samples for microbial analysis were collected immediately on arrival at the laboratory and stored at -20 °C before DNA extraction. Microbial analyses were only carried out on the starting inocula since preliminary testing (data not shown) showed that the microbial community does not change during the inhibition testing regardless of the TAN composition. This is likely due to the short test duration (~1.5 days).

Genomic DNA was extracted from each inoculum by FastSpin for Soil Kit (MP-Biomedicals, USA) according to manufacturer's protocol. Pair-end 16S amplicon sequencing was conducted by the Australian Centre for Ecogenomics at The University of Queensland (Australia) with primer sets 926F (5'-AAACTYAAAKGAATTGACGG-3') and 1392wR (5'-

ACGGGCGGTGWGTRC-3') [31] by Illumina Miseq Platform. Library preparation was performed according to the Illumina workflow (#15044223 Rev.B). Trimming and quality control were performed on raw paired reads with Trimmomatic [32], Pandaseq [33] and FASTX-Toolkit [34]. The joined high-quality sequences were analysed by QIIME (v1.9.0) [35] and singlet reads were removed from the operational taxonomy units (OTUs) table.

The raw OTUs table was imported into R (v3.2.3) and rarefied to 12,000 sequences per sample by function "rarefy_even_depth" of package phyloseq with default settings [36]. The rarefied OTU table can be found in the supplementary material. Hellinger transformed OTUs tables were used for Principle Component Analysis (PCA) by function "rda" in the package vegan, to determine overall community characteristics. PCA directly on rarefied OTUs tables gave similar results, but commonly with weaker correlations. In order to assess inhibition relationships completely, PCA was done separately for the bacterial, archaeal and overall communities. Rarefied overall OTUs table was then summarised at the lowest possible taxonomic level with >5% relative abundance used to generate the heatmap.

Inhibition testing

Inhibition testing was performed following the rapid inhibition method (~1.5 days) developed by Astals et al. [20]. Inocula with VS concentration above 10 $gVS \cdot L^{-1}$ were diluted to 10 $gVS \cdot L^{-1}$ with deionised water, while inocula with VS concentration below 10 $gVS \cdot L^{-1}$ were not diluted. The main reason for dilution was to standardise concentrations and reduce matrix effects from the host environments. This allowed to isolate the impact of the microbial community composition. However, the inhibition results may not strictly represent performance in the full-sale process (e.g. inhibitors interaction). The inhibition tests were performed in 160 mL serum bottles containing inoculum (99 mL), the amount of ammonium

chloride needed to reach the design TAN concentration, and the amount of acetate solution needed to reach an inoculum-to-acetate ratio of 5. Acetate was selected as carbon source since it can be used by the two major methane generation pathways, i.e. acetoclastic methanogenesis and acetotrophic (syntrophic acetate oxidation followed by hydrogenotrophic methanogenesis). Ammonium chloride was selected as TAN and NH_3 source since it has been the most used reagent to simulate ammonia inhibition [6, 13, 14, 20, 27, 37, 38]. Each inoculum was tested at seven different TAN concentrations (added at 0, 1, 2, 3, 5, 10 and 15 $gN \cdot L^{-1}$). A control test (no added ammonium chloride) was run for each inoculum. pH of each bottle was measured once all reagents were added (pH was never adjusted). The headspace of each test bottle was flushed for 1 min (4 $L \cdot min^{-1}$) with 99.99% nitrogen gas before sealing the serum bottle with a rubber stopper and aluminium crimp. The gas line was not submerged during flushing. Subsequently, the serum bottles were placed in a temperature-controlled incubator at 37 ± 1 ºC for ambient and mesophilic inocula and at 55 ± 1 ºC for the thermophilic inoculum.

The SMA of the inoculum at each concentration was determined through three sampling events (0.5, 1.0 and 1.5 days), where biogas headspace pressure (bench-top manometer) and biogas composition (gas chromatography) were quantified. Accumulated volumetric methane is expressed in grams of chemical oxygen demand (gCOD) under standard conditions (0 ºC, 1 bar). All conditions were tested in triplicate. SMA was determined as the slope of a linear regression (Analysis Toolpak in Microsoft Excel 2013) applied to the cumulative methane production (y-axis) and time (x-axis), for subsets of data over which the rate of methane production was approximately constant. Inhibition profile plots of SMA vs. NH_3 concentration were normalised against the maximum SMA (control test, SMA without added ammonium chloride). The slope of the inhibition curve ("speed of inhibition") was determined using the Microsoft Excel built-in function "LINEST", applied to subsets of data over which the rate of

inhibition increase was approximately constant. Standard errors in slopes were analytically calculated by the Microsoft Excel tool from the regression standard error. The 95% confidence interval in slope was determined using a two-tailed t-test with n-2 degrees of freedom where n is the number of data points available for regression.

The NH_3 KI_{50} of each inoculum was quantified by determining the NH_3 concentration at which measured SMA is reduced by 50% of the maximum measured SMA. This was determined by linear regression (Analysis Toolpak in Microsoft Excel 2013) through the linear section of the NH_3 vs. SMA curve, and determination of the NH_3 concentration where the SMA was 50% of the maximum from this regression. Standard error and confidence interval in KI_{50} was determined from the standard error in regression as above.

Statistical and correlation analysis

Analysis of variance in mixed categorical/continuous mode (ANCOVA) was done to test significance of factors on both microbial community and NH_3 resilience (assessed as KI_{50}) using the function "anovan" of Matlab (MATLAB version R2014b). Specifically, PC values of bacterial, archaeal and overall communities were separately used as output with numerical variables pH, TAN and NH_3, treated as continuous factors, temperature as a coded continuous factor (values of '1', '2', or '3' assigned to ambient, mesophilic and thermophilic, respectively), and substrate type as a categorical factor. The full model was tested followed by elimination of non-significant factors to the minimum-parameter, most parsimonious model. Likewise, SMA, slope of the inhibition curve and KI_{50} were tested as outputs against factors as above, but also against PC scores and relative abundance of archaea (both as continuous factors). A significance threshold of 0.05 was applied for rejection of the null hypothesis.

RESULTS

Chemical characterisation

Table 2 contains selected characteristics of the 13 inocula under study. pH values ranged from 7.2 (approximately neutral) to 8.4. Most inocula, except Sludge_4 and Slurry_3, had TAN concentrations below 1000 mgN·L^{-1}. Sludge_4 (with thermal hydrolysis pre-treatment) had the highest TAN (2833 mgN·L^{-1}) and NH_3 (241 mgN·L^{-1}) concentration. Leachbed reactor inocula (LBR_1 and LBR_2) and Slaughter_2 (the only thermophilic system) had NH_3 concentrations higher than 100 mgN·L^{-1}. Slaughter_1, Slurry_1, Slurry_2 (all running at ambient) and both Granule inocula had NH_3 concentrations below 40 mgN·L^{-1}. Slurry_3 that operated at pH 7.8 and a mesophilic temperature had a comparatively moderate NH_3 concentration (87.5 mgN·L^{-1}).

The NH_3 concentrations of the inocula under study (0.1 – 241 mgN·L^{-1}) were considered in the low and moderate range but representative of most large scale applications [2, 28, 39, 40]. Significantly higher NH_3 background concentrations (>300 mgN·L^{-1}) are commonly observed in manure and food waste digesters, especially those operating at thermophilic conditions [18, 41, 42]; nonetheless, such AD systems are not common in Australia.

Microbial characterisation

Methanogens relative abundance ranged between 9% and 64% for the different inocula (Table SI in the supplementary material). The abundance of methanogens was highest in the Granule inocula (>60%). Methanogen abundance was also high in the Slaughter inocula (>50%), and relatively low in the LBR inocula (18-23%). The relative abundance of methanogens in the Sludge and Slurry inocula was more variable with values ranging 9-47% and 22-36%, respectively. *Methanosaeta* was the dominant methanogen in the Sludge and Slurry inocula,

the LBRs inocula were dominated by *Methanosarcina*, while the relative abundance of *Methanosaeta* (acetoclastic methanogen) and a pool of hydrogenotrophic methanogens was similar in the Slaughter and Granule inocula (Figure 1). The dominant hydrogenotrophic methanogens in Slaughter and Granule inocula were *Methanobacterium* and *Methanothermobacter*. A number of *Methanosaeta* OTUs were identified, with 7 inocula dominated by a single common OTU (named *Methanosaeta_1*), while Sludge_1, Sludge_4, Slaughter_2 and Granule_1 were dominated by other *Methanosaeta* OTUs. Granule_1, Sludge_1 and Slaughter_1 had a diverse *Methanosaeta* population (Figure 2).

Bacterial communities consisted mainly of OTUs from the phylum *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Proteobacteria* (Figure 1). *Bacteroidetes* and *Chloroflexi* were abundant in all the tested inocula, *Firmicutes* was particularly dominant in Slurry inocula, and *Proteobacteria* was mostly found in Sludge and Granule inocula.

Inhibition testing

SMA values from the control tests (test without added ammonium chloride) are shown in Table 2 and ranged from 0.04 (Slurry_2) to 0.21 gCOD-CH_4·g VS^{-1}·day^{-1} (Granule_1). KI_{50} values ranged from 32 to 175 mgNH_3-N·L^{-1} (Sludge_2 and Granule_1, respectively), indicating moderate variability in terms of NH_3 resilience when compared to variability reported in the literature [8, 9]. KI_{50} values varied across substrates and within a substrate type (Figure 3). For instance, KI_{50} values of Sludge and Slurry inocula varied from 32 to 138 mgNH_3-N·L^{-1} and from 75 to 169 mgNH_3-N·L^{-1}, respectively. Regarding the shape of the inhibition threshold curves, most inocula were inhibited as soon as the NH_3 concentration increased, i.e. the inhibition curve had a concave shape. However, three inocula (Sludge_3, LBR_1 and LBR_2)

showed a sigmoidal shape with a range of NH_3 concentrations over which inhibition did not drastically change (Figure 3).

Factors affecting microbial community and inhibition resilience

Substrate type was the strongest factor correlated with microbial community principal components, significantly with overall community PC1 (p=9×10^{-4}) and PC2 (p=6×10^{-4}) and bacterial community PC1 (p=2×10^{-7}) and PC2 (p=3×10^{-5}). Substrate type was also a predictor for archaeal community PC1 (p=0.035, considering also temperature as a predictor) and archaeal community PC2 (p=0.025, with no other predictors). Temperature was the most significant predictor for archaeal community PC1 (p=0.018), and also a predictor for overall community PC1 (p=0.015) when considered in conjunction with the dominant predictor of substrate type. Archaeal community PC1 was significantly correlated with pH and NH_3, only when considered in isolation (p=0.001 and 0.007 respectively), but not when the primary factors of substrate type and temperature were also included.

A plot of major archaeal community ranked from the most to least resilient to NH_3 (i.e. highest to lowest KI_{50} value) is shown in Figure 2. A KI_{50} value of 100 mgNH_3-N·L^{-1} was selected as threshold between the low and the high resilience inocula since the average and the median KI_{50} of the inocula under study were 105 and 110 mgNH_3-N·L^{-1}, respectively. Inocula collected from digesters with lower NH_3 concentration (<40 mgN·L^{-1}) generally had KI_{50} below 100 mgNH_3-N·L^{-1} and were dominated by a single OTU affiliated with *Methanosaeta* (*Methanosaeta_1*). Sludge_1 and Granule_1 inocula were also collected from digesters with a low NH_3 background concentrations; however, their methanogenic community contained multiple OTUs affiliated with *Methanosaeta* rather than dominated by *Methanosaeta_1*.

Archaeal communities were more variable in inocula with KI_{50} higher than 100 mgNH_3-N·L^{-1}. Slurry_3 was dominated by *Methanosaeta_1*, while Granule_1 and Sludge_4 had OTUs affiliated with different *Methanosaeta*. Slaughter_2 and Granule_2 archaeal community had *Methanosaeta* and hydrogenotrophic methanogens (*Methanothermobacter* and *Methanobacterium*), while LBRs inocula where dominated by *Methanosarcina*. It is worth mentioning that the highest KI_{50} was not obtained from the inocula with the highest background NH_3 concentration (Sludge_4 and LBR_2), but rather with Granule_1 and Slurry_3 collected from digesters with NH_3 concentrations of 0.1 and 87.5 mgN·L^{-1}, respectively.

Statistically, no microbial factors or other operational factors were correlated with NH_3 resilience, as quantified by KI_{50} values. The total relative abundance of archaea (Table SI) also did not appear to affect inhibition resilience (p=0.5369). Archaeal relative abundance and archaeal community PC2 were the main predictors for specific methanogenic activity (p=0.0107 and p=0.0242 respectively), with archaeal community PC2 primarily correlated with substrate type. However, substrate type was not correlated with methanogenic activity. In addition, substrate type (p=0.01), temperature (p=0.02) and NH_3 (p=0.005) were significantly correlated to the slope of the inhibition curve when considered in conjunction, while the bacterial community PC1 was correlated to the slope of the inhibition curve only when considered in isolation (p=0.0119). However, significance was dependent on LBR_1 for which a certain threshold NH_3 concentration caused a sharp decrease in activity (increase in inhibition). When LBR_1 was omitted, the PC1 significance was lost.

DISCUSSION

Substrate type and temperature were the primary factors influencing the microbial community. Overall community PCA variation was completely driven by the archaeal shifts, with similar

correlations in both overall community and archaeal community PC1 to substrate type and temperature. The relationship between bacteria and substrate type was driven by a distinct bacterial community according to substrate type (clearly seen by heatmap hotspots in Figure 1), while the archaeal community shift with substrate type was mainly driven by a shift away from *Methanosaeta_1* towards a more diverse methanogenic population (including other *Methanosaeta* OTUs). This shift in the archaeal community was unrelated to inhibition resilience as quantified by KI_{50}, but was related to specific methanogenic activity. pH and NH_3 should be considered as secondary rather than primary factors for the archaeal community, because significance was lost when variance was proportioned to substrate type or temperature.

Methanosaeta was the dominant archaea genus in inocula with resilience above and below 100 mgNH_3-N·L^{-1} (Figure 2). Possible reasons are:

(i) At low and moderate NH_3 background concentrations (0.1 – 241 mgN·L-1), NH_3 is not a key driving factor of the microbial profile, in contrast to what is observed at higher NH_3 levels [26, 27, 37].

(ii) NH_3 resilience may not be reflected directly by microbial community profile, as shifts of microbial community are decoupled from microbial functions. This is suggested by Zhang et al. [14] who reported a strong transcriptional response to NH_3 stress without a change in the community structure.

Hydrogenotrophic methanogens (e.g. M*ethanomicrobiales* and M*ethanobacteriales* order) were not present in inocula with higher NH_3 background concentrations (Figure 1 and 3), although the acetotrophic pathway (syntrophic acetate oxidation followed by hydrogenotrophic methanogenesis) has been reported to be the dominant methanogenic pathway at high NH_3 concentrations [18, 27, 37]. The occurrence of *Methanothermobacter* in Slaughter_2 may have

been a consequence of thermophilic conditions [43], since Slaughter_1 and Slaughter_2 were treating wastewater from the same slaughterhouse (albeit using different pre-treatment and different AD technology) and *Methanothermobacter* was not detected in Slaughter_1. Slaughter_2 also had higher resilience to ammonia than Slaughter_1, which is in agreement with Wiegant & Zeeman [44] who reported higher NH_3 resilience of acetoclastic methanogens at thermophilic conditions when compared to mesophilic conditions.

In the present study, the inhibition resistance for granules was variable (118-175 mgNH_3-N·L^{-1}), but generally toward the higher range of resilience for the inocula tested. High inhibition resilience has also been reported for anaerobic granules as compared to un-acclimated non-granular inoculum, including resilience to NH_3 inhibition [45, 46]. Methanogens are typically not located on the surface of anaerobic granules [47] and resilience has previously been credited to internal diffusion resistance reducing the concentration of NH_3 within the granule structure [48]. Specific NH_3 diffusion rates are unknown, however, the high diffusion coefficient of ammonia, the fact that ammonia is only consumed by anabolism and the relatively large time-scale of SMA testing (i.e. ~1.5 days) are likely to have resulted in a reasonably uniform NH_3 concentration throughout granules. This was further supported by the granules inocula inhibition profile (Figure 3) where no threshold concentration prior to inhibition was observed. The onset of inhibition at low NH_3 concentrations implies that there was no significant NH_3 gradient within the granules and therefore, the stronger ammonia resilience in granules is unlikely to be linked to diffusion.

LBR inocula were dominated by *Methanosarcina* and had a mid-range NH_3 resilience (KI_{50} = 110 mgNH_3-N·L^{-1}) within the inocula under study. Yap et al. [29] linked the presence of *Methanosarcina* in LBRs to the high concentration of volatile fatty acid (VFA) in the digester

(up to 3.5 $g \cdot L^{-1}$) rather than due to resilience to NH_3. The inhibition profiles of LBRs indicated that *Methanosarcina* could tolerate a certain NH_3 concentration before notable inhibition was observed (Figure 3). This behaviour could be attributed to the lower surface-to-volume ratio of *Methanosarcina* and its ability to form clusters [16, 45].

A negative correlation between archaeal community PC2 and SMA (noting the underlying factor of substrate type) is interesting, with the shift (towards lower PC2, higher SMA) driven by the emergence of a diverse community of mainly *Methanosaeta* and *Methanobacteria*, instead of the otherwise dominant *Methanosaeta*_1. Furthermore, methanogenic activity was also impacted by the relative abundance of total archaea. The correlation between SMA and methanogens relative abundance was obtained, despite SMA values being affected by the inoculum background VS concentration. However, the fact that methanogenic activity was correlated to total relative abundance rather than an individual OTU suggests that different methanogens can have similar activity. The normalisation of the SMA testing is a topic of current discussion within the anaerobic community.

Overall, the fact that no operational or microbial factors led to higher NH_3 resilience and that the slope of the inhibition curve was dependent on several factors (i.e. substrate type, temperature and NH_3 concentration) suggest that, for the tested NH_3 range of 0.1 – 241 $mgN \cdot L^{-1}$, there is minimal opportunity to develop engineered solutions to enhance microbial resilience to ammonia. The results also suggest that at low and moderate NH_3 background concentrations inhibition resilience and microbial profile cannot be related, and thus inhibition resilience needs to be individually tested for specific digesters.

CONCLUSIONS

Across 13 anaerobic inocula tested, overall microbial community was mainly driven by substrate type and temperature of the anaerobic digesters from where the inocula were sampled. pH value and NH_3 showed secondary effects. NH_3 resilience (measured as KI_{50}) was highly uniform across the tested inocula and could not be correlated to any of various tested factors. However, the slope of the inhibition curve was influenced by environmental factors, including substrate type, temperature and NH_3, and weakly correlated to microbial community. This work suggests that, with relatively low background NH_3 concentrations, neither community nor physical factors are key determinants of NH_3 resilience.

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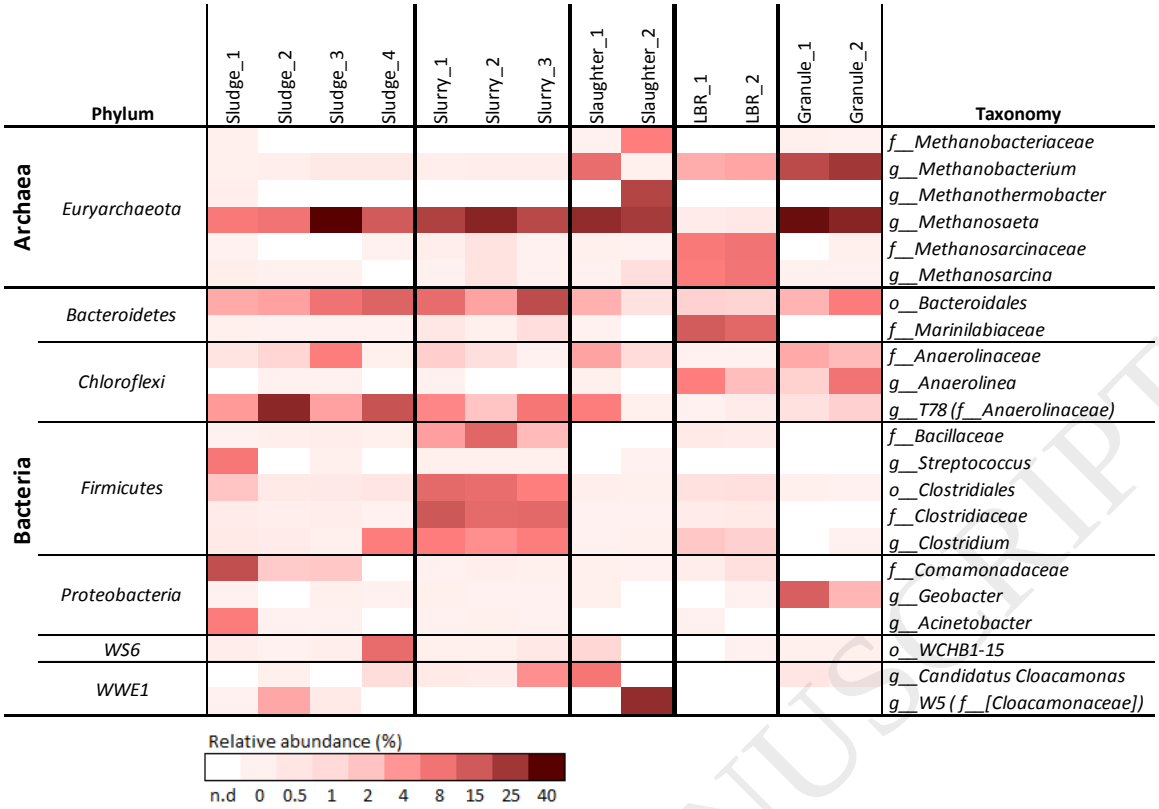

Figure 1. Heatmap showing the microorganisms with relative abundance above 5% for the inocula under study

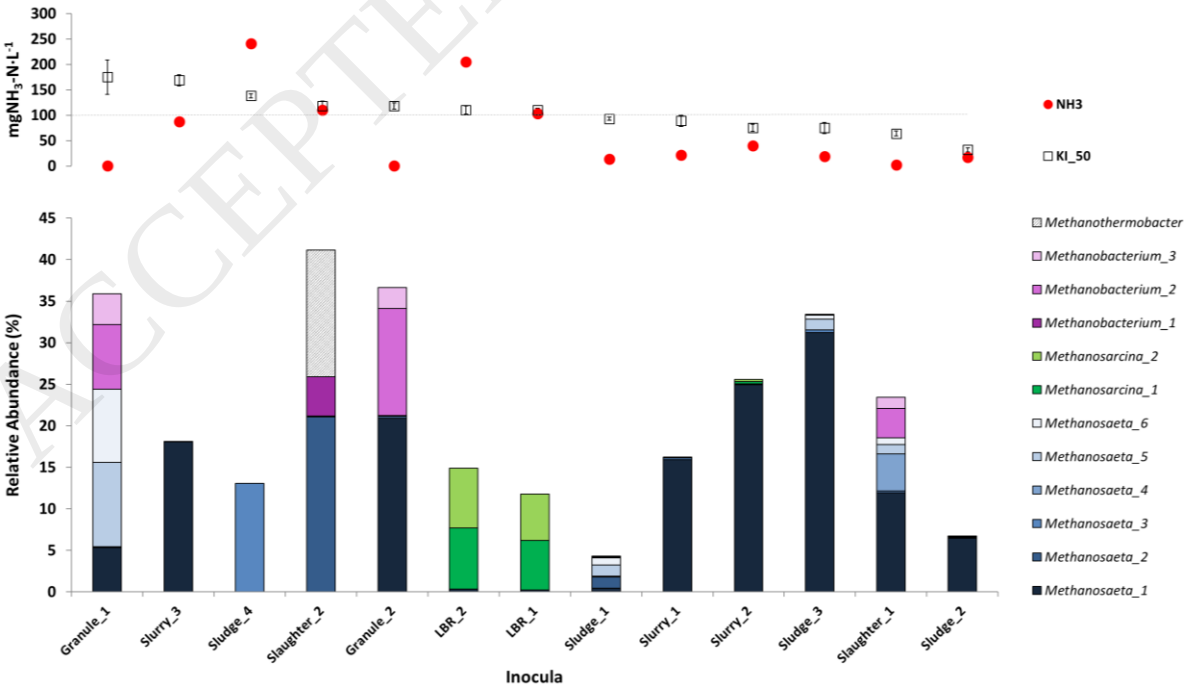

Figure 2. Bottom chart shows the major archaeal OTUs abundance (>1%, as bar chart). Upper chart shows the inoculum background NH_3 concentration (red marker) and KI_{50} (box marker) for the inocula under study. Samples are organised in descending KI_{50} values.

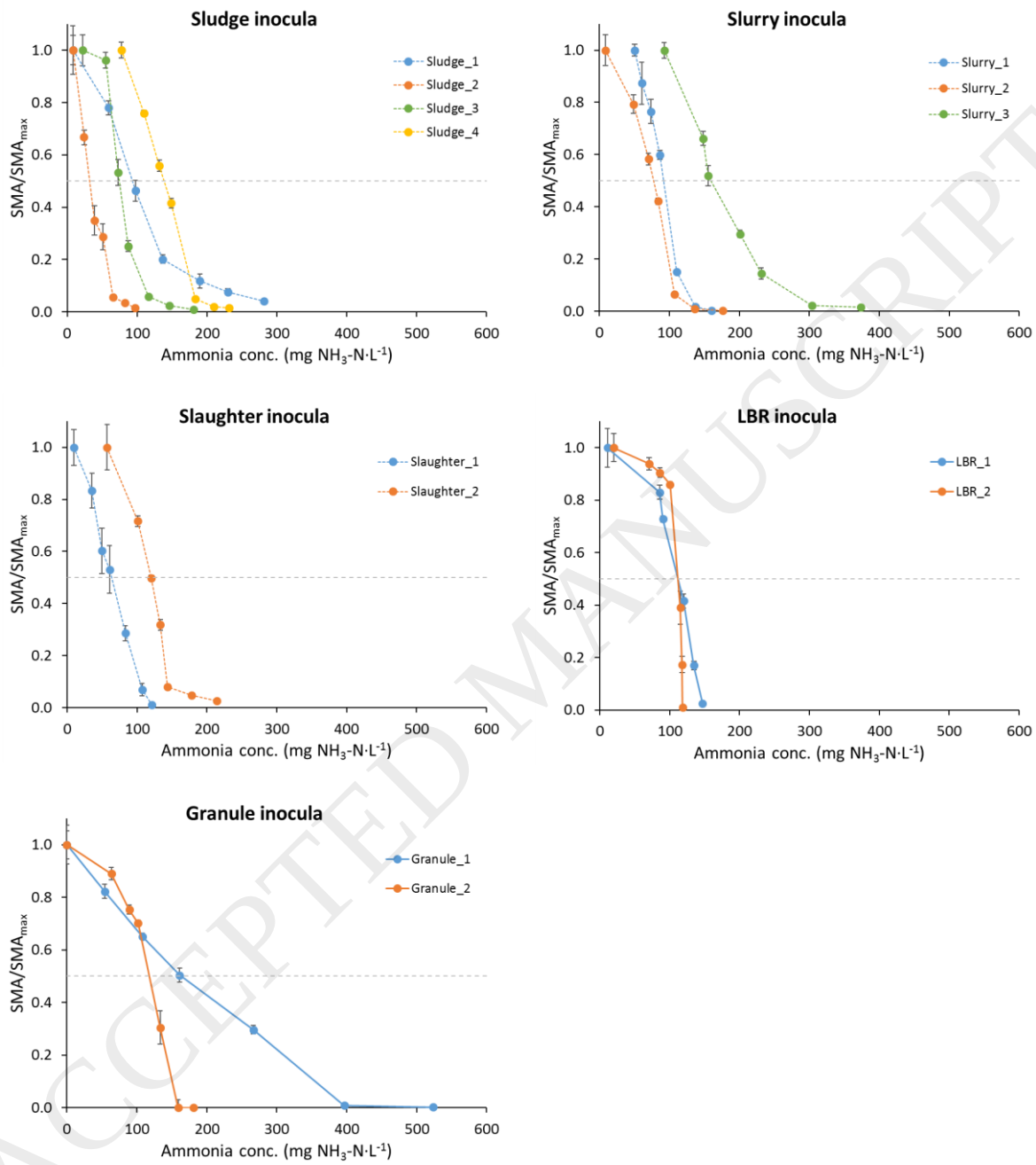

Figure 3. Normalised (SMA/SMA_{max}) inhibition threshold curves for the inocula under study. The grey dotted line indicates where the activity is half the maximum, from which the KI_{50} of each inoculum was obtained.

Table 1. Operational conditions of the anaerobic digesters from where inocula were collected

Inocula	Substrate	Digester Type	Temperature
Sludge_1	Sewage sludge	CSTR	Mesophilic
Sludge_2	Sewage sludge	CSTR	Mesophilic
Sludge_3	Sewage sludge	CSTR	Mesophilic
Sludge_4	TH WAS*	CSTR	Mesophilic
LBR_1	Crop/manure mix	Batch solid phase leachbed - Pilot (Flood & Drain)	Mesophilic
LBR_2	Crop/manure mix	Batch solid phase leachbed - Pilot (Trickling)	Mesophilic
Slaughter_1	Slaughterhouse wastewater	Covered Pond	Ambient
Slaughter_2	Slaughterhouse wastewater	AnMBR** - Pilot	Thermophilic
Slurry_1	Pig slurry	Covered Pond	Ambient
Slurry_2	Pig slurry	Covered Pond	Ambient
Slurry_3	Pig slurry	Mixed in-ground digester	Mesophilic
Granule_1	Soluble organic wastewater	UASB***	Mesophilic
Granule_2	Soluble organic wastewater	UASB	Mesophilic

*TH WAS: thermally hydrolysed waste activated sludge
**AnMBR: anaerobic membrane bioreactor
***UASB: upflow anaerobic sludge blanket reactor

Table 2. Characteristics of the inocula under study

Inocula	pH	VS	TAN	NH_3	KI_{50}	SMA*	Inhibition slope
		$g \cdot kg^{-1}$	$mgN \cdot L^{-1}$	$mgN \cdot L^{-1}$	$mgNH_3\text{-}N \cdot L^{-1}$	$gCOD\text{-}CH_4 \cdot gVS^{-1} \cdot day^{-1}$	$gCOD\text{-}CH_4 \cdot L \cdot gVS^{-1} \cdot day^{-1} \cdot mgNH_3\text{-}N^{-1}$
Sludge_1	7.7	16.6	268	13.9	93 ± 3	0.061±0.003	-0.0004±0.0002
Sludge_2	7.2	25.9	953	17.4	32 ± 4	0.050±0.005	-0.0010±0.0002
Sludge_3	7.3	15.8	949	18.9	75 ± 11	0.132±0.008	-0.0029±0.0021

Sludge_4	7.9	29.3	2833	241.3	138 ± 4	0.075±0.002	-0.0007±0.0001
Slurry_1	7.7	9.3	776	21.7	89 ± 11	0.075±0.002	-0.0010±0.0003
Slurry_2	8.1	97.6	588	39.6	75 ± 7	0.035±0.003	-0.0005±0.0002
Slurry_3	7.8	9.3	1384	87.5	169 ± 11	0.093±0.003	-0.0006±0.0001
Slaughter_1	7.3	8.2	255	2.6	64 ± 5	0.136±0.009	-0.0014±0.0003
Slaughter_2	8.0	11.4	365	110	118 ± 10	0.138±0.032	-0.0014±0.0007
LBR_1	8.1	96.5	803	103	110 ± 4	0.101±0.007	-0.0013±0.0004
LBR_2	8.4	89.9	985	205	110 ± 8	0.068±0.004	-0.0028±0.0025
Granule_1	6.7	n.d.**	20	0.1	175±34	0.206±0.012	-0.0005±0.0001
Granule_2	7.1	n.d.	18	0.3	118±6	0.128±0.003	-0.0016±0.0003

* SMA from the control test, i.e. without added ammonium chloride

** non-determined. Granules VS were determined after washing them over a 200 µm mesh sieve to remove granule debries